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Muscle Differentiation: How Two Cells Become One

Review

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A key feature of myogenesis is the fusion of myoblasts to form multinucleate myotubes. Recent work in Drosophila has uncovered a collection of genes that operate at different stages of this process. Some interactions between them have been described that begin to define links from outside the cell via the plasma membrane to the cytoskeleton. Future studies will establish the extent to which the molecular mechanisms of myoblast fusion are conserved between Drosophila and other animals, as found in other aspects of myogenesis.

Introduction

One fascinating feature in the formation of muscle is the fusion of myoblasts to make multinucleate syncytia. The interest in this phenomenon is sparked both by a desire to understand this characteristic and unusual aspect of muscle cell biology and by a hope that fusion may be a route for new therapies for a range of diseases [1]. Studies in *Drosophila melanogaster* have proved fruitful in the analysis of many aspects of the molecular genetics and cell biology of muscle development, and over the last few years there has been considerable progress in the analysis of myoblast fusion (reviewed in [2–4]). Work published in a recent clutch of papers has advanced the field still further, both conceptually and in the identification of the molecular players [5–15]. This review concentrates on the findings of these recent studies.

The Story So Far

The formation of the *Drosophila* mesoderm and its subsequent subdivision into groups of cells that will differentiate in a particular way has been extensively analysed [16–21] and reviewed [22–24]. One group of these cells is the somatic mesoderm that differentiates to form the somatic muscles of the body wall. There is a stereotypic pattern of thirty such muscles of different sizes and shapes in each hemisegment of the embryo. These muscles are the principle focus of this review and have many similarities to vertebrate skeletal muscle. They form in the following way. Within the somatic mesoderm, muscle progenitors are singled out from their neighbours by lateral inhibition in a similar *Notch*-regulated process to that used in neurogenesis (Figure 1A). Progenitors then divide to form 'founder cells' (Figure 1B). Founders endow the developing muscles with their specific characteristics through the expression of so-called 'muscle identity' genes (reviewed in [23–25]). An individual founder then

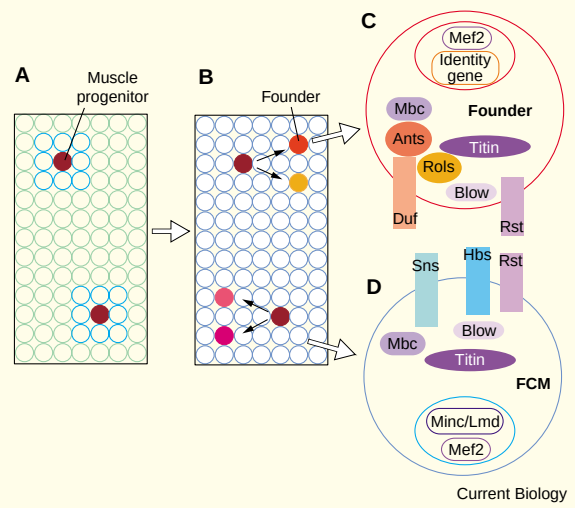

Figure 1. The development of founders and fusion competent myoblasts (FCMs).

(A) Formation of two muscle progenitors (brown) through lateral inhibition from two clusters of somatic mesoderm cells in one hemisegment of the developing embryo. (B) Muscle progenitors (brown) divide to form two founder cells (shades of red). FCMs are shown in blue. (C,D) Distribution of proteins in founder cell (red) and FCM (blue). There are three sets of molecules: surface, cytoplasmic and nuclear. Some are asymmetrically distributed: Duf, Ants, Rols and 'identity genes' in founders (shades of red); and Sns, Hbs and Minc/Lmd in FCMs (shades of blue). Other proteins are in both cell types and include Rst, Mbc, Blow, Titin, Mef2 (shades of purple). Additional proteins implicated in fusion, but not shown, include the Rho-like GTPase Drac1 [41] and the SH2/3 adaptor protein, D-Crk [42].

attracts and fuses with the other cell type derived from the somatic mesoderm, the Fusion Competent Myoblasts (FCMs), and recruits them to its pattern of muscle identity gene expression (Figure 2). Initially, muscle precursors with two or three nuclei are formed, which enlarge by further fusion to form the mature individual multinucleate myotubes (Figure 2). Fusion is asymmetric: founders fuse with FCMs, but neither myoblast type fuses with itself [26,27].

A framework for understanding aspects of somatic muscle fusion is provided by the studies of Bate and of Doberstein *et al.* [28,29]. The former analysis revealed when and where myoblast fusion occurs, described the formation of muscle precursors with extensive fine processes, and provided evidence for founder cells. The latter revealed many distinct steps in the fusion process, including ultrastructural detail at the membrane through electron microscopic analysis, and established that different genes act at different stages in myoblast fusion. Examples of genes whose mutant phenotypes are disruption of myoblast fusion at different stages include *myoblast city* (*mbc*), *Dmef2*, *blown fuse* (*blow*) and *Dtitin* [29–33]. Each protein product is found in both classes of myoblasts, the founders and the FCMs.

Considerable interest was sparked by the discovery of two other genes, *dumbfounded* (*duf*) and *sticks and stones* (*sns*) [34,35]. Both genes encode immunoglobulin superfamily members with the structure of a transmembrane adhesion or signalling molecule. They too affect myoblast fusion, but unlike the molecules known previously, are specifically expressed: *duf* only in founders, and *sns* only in FCMs. This was striking because of the known asymmetry of the fusion process. A working model emerged in which FCMs, characterised by *sns* expression, move towards founders in response to Duf, which had been shown to function as a myoblast attractant. Fusion then proceeds with symmetrically distributed cytoplasmic or membrane-associated proteins like Mbc, Blow and D-Titin functioning on both sides of the membrane.

The model had few details. It also raised many questions: are there other founder-specific or FCM-specific molecules? How do the players interact and co-operate in fusion? Are cell surface molecules other than Duf and Sns involved? Does Duf function at levels other than as an attractant? And do *duf* and *sns* function in systems other than *Drosophila* somatic muscle fusion? Some answers to these questions are provided by the recently reported analyses reviewed here that include the descriptions of novel genes identified in a variety of screens [5–15].

New Cytoplasmic Proteins

The answers begin with two genes isolated in independent screens for genes that affect muscle development. They are *antisocial* (*ants*) and *rolling pebbles* (*rols*) [5–7]. Mutation in either gene blocks myoblast fusion and results in no multinucleate myotubes. In contrast to *duf* and *sns*, both *ants* and *rols* encode proteins that are cytoplasmic and appear to become enriched at the plasma membrane during fusion. Each has multiple protein–protein interaction domains and these are organised in a remarkably similar way: at the amino terminus is a RING finger, and at the carboxyl terminus there are nine ankyrin repeats followed by three tetratricopeptide repeats (TPRs) and coiled coil. Chen and Olson [5] then investigated the proteins with which Ants might interact. They undertook a series of immunoprecipitation assays in S2 cultured cells co-transfected with tagged Ants together with tagged Blow, Sns, Duf or Mbc. No interaction of Ants with Blow or Sns was detected. However, Ants did interact with both Duf, the transmembrane protein, and Mbc, a cytoplasmic protein implicated in mediating changes in the cytoskeleton [36]. This is the first direct evidence that specific proteins identified for their role in myoblast fusion physically interact.

The significance of this work is that Ants may be part of a protein complex that links the membrane to the cytoplasm and which may integrate the initial myoblast attraction event with cytoskeletal reorganisation in the developing myotube. Given its structural similarities to Ants, Rols too may be involved in the same or a similar complex. There is no direct evidence, but some findings are consistent with the idea (see below). However, one tantalising glimpse of some other function for *rols* is the presence of a lipolytic enzyme motif [6]. Although its role in Rols has not been explored directly, an indication that it may be significant comes from the analysis of two Rols isoforms, 6 and 7, produced through alternative splicing [6,7]. Rols7 can rescue the *rols* mutant phenotype and contains the lipolytic motif, whereas Rols6 does not contain this motif and cannot rescue the mutant [6].

The expression patterns of *ants* and *rols* are striking. In common with *duf*, both *ants* and *rols7* are expressed in founders and not FCMs, and their temporal expression broadly coincides with the fusion process [6,7]. There are two points of interest here. First, it had been suggested that there might be founder cell-specific genes in addition to *duf* because of the observation that expressing Duf in the FCMs did not make these cells fuse with each other [34]. So, this finding was anticipated, although it has been found that co-expression of Rols with Duf is also not sufficient for ectopic myoblast fusion [6]. Second, the discovery of founder-specific cytoplasmic proteins indicates that the asymmetry of the fusion process extends to the molecular machinery inside the founder cell and does not simply reside with surface molecules (Figure 1C,D).

In their analysis of *rols,* Rau *et al.* [7] highlighted another aspect of myoblast fusion that hitherto has not received much attention. Muscle precursors are formed by the initial fusion of the founder with one or two FCMs. Precursors then grow by fusion. The question is whether or not there are distinct genes required for these sequential steps. From a careful analysis of the *rol*s mutant phenotype it is apparent that *rols* is not required for the initial fusion, but is required in the muscle precursor for the subsequent recruitment of further FCMs to form the multinucleate myotube [6,7]. A requirement at this stage contrasts with other mutants, such as *mbc*, in which the block is earlier and almost no fusions occur (Figure 2) [30,37]. At present it isn't known how earlier fusion events may differ from later ones.

Fusion Competent Myoblasts — Independent and Non-Uniform

Understanding fusion requires an in depth knowledge of both partners in the process. One of them, the FCMs, have been relatively unexplored and generally have been thought of simply as a uniform population of myoblasts that were not selected to be founders. However, two recent papers ensure that they will now attract more attention [8,9]. Analysis of a gene called *myoblasts incompetent* (*minc*) or *lame duck* (*lmd*) shows that the FCMs have their own differentiation program and also appear not to be a uniform population. *minc*/*lmd* was identified in two screens, one for genes affecting myogenesis, the other for proteins that bind a *mef2* enhancer. Incidentally, the same gene has also been identified in two expression based screens [38,39].

In *minc*/*lmd* mutants the founders differentiate normally, but there is a failure in FCM differentiation and there is no myoblast fusion at all [8,9]. Unexpectedly, this indicates that the FCMs have their own differentiation program separate from that in founders.

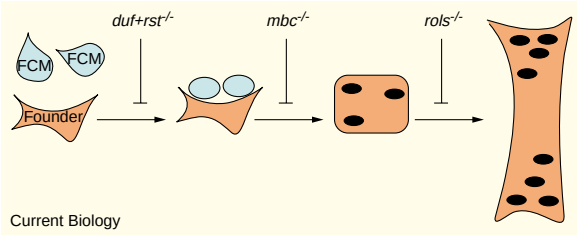

Figure 2. Stages of myoblast fusion.

Myoblast fusion is a multistep process. First, FCMs (blue) are attracted towards founder cells (red). In *duf*, *rst* double mutants the FCMs are not attracted and their filopodia extend in random orientations rather than towards the founders [34]. Second, FCMs associate with the founders. They then fuse to form bi- or tri-nucleate muscle precursors and are recruited to the founder's pattern of gene expression. This step is blocked in *mbc* mutants [30,37]. Lastly, in a process that requires *rols*, additional FCMs fuse with the muscle precursors to form multinucleate myotubes [6,7]. For clarity only a few gene functions and some of the steps are shown.

minc/lmd encodes a putative zinc finger transcription factor related to vertebrate Gli proteins and ascidian Macho1 [8,9], and may therefore control expression of a group of genes required for FCM differentiation. It is apparent that *minc/lmd* has roles in steps common to all myogenic cells, such as *mef2* expression, as well as in steps specific to FCMs, for example *sns* expression [8,9].

In the somatic mesoderm *minc/lmd* is expressed in immature myoblasts, predominantly in the FCMs. However, and unexpectedly, the FCMs are found to be non-uniform with respect to the nuclear localisation of Minc/Lmd [8]. Nuclear localisation correlates with expression of *mef2* and it is suggested that nuclear Minc/Lmd is required for the expression of *mef2*. This non-uniformity is consistent with an earlier suggestion that the differentiation state of cells competent for fusion may be time-dependent [2]. An additional facet to this may be the temporal and spatial expression in FCMs of another gene, *hbs* (see below), which led to the suggestion that there may be undescribed functional differences within the FCM population [12]. Taken together these observations indicate that the FCMs are not a uniform population and, more speculatively, that all fusions may not be equal: earlier could be distinct from later.

Another aspect to a lack of uniformity within the FCMs is the idea, which has been considered for some time, that the cells that fuse together to make one muscle are distinguishable from those that fuse to make a distinct, nearby muscle. This is certainly one way in which muscle development could be organised. In fact there is no evidence for a patterning mechanism that selects myoblasts in groups assigning them to make a specific muscle. Rather, patterning mechanisms govern the founders and these then recruit FCMs [34]. Notwithstanding this, one could imagine that the myoblasts that fuse to make an individual muscle are clonally related, as they develop in close proximity. A recent elegant study has shown that they do not **have** to be. Klapper *et al.* [10] have developed a technique that allows them to detect syncytia. The technique in which they produce genetic mosaics of cells is general and does not apply just to muscle. They transplant single cells carrying a UAS–reporter gene construct into a recipient embryo that carries a constitutively active GAL4 gene. The GAL4 transcription factor will activate the reporter gene through binding to the UAS motif only when cells of the two genotypes fuse. When they apply the technique to muscle development, they find activation of the reporter in most somatic muscles, although not all, and in this way show that non-clonally related myoblasts can fuse with each other. They conclude that clonal relationships generally seem to play no crucial role in the selection of the participating cells.

Taken together the available evidence indicates that fusion between founders and FCMs, and later between precursors and FCMs, simply occurs with the nearest FCMs. Given such a mechanism, an immediate question to ask is how do each of the 30 different muscles in each hemisegment end up different specific sizes. The smallest contain just 3–4 nuclei, the largest 20–25 [28]. Current ideas suggest that size is one characteristic of an individual muscle governed by the muscle identity genes. A component of a possible mechanism would therefore involve the regulation by muscle identity genes of one, or probably more, of the molecules critical to fusion. The transient expression, correlating with fusion, of *duf*, *rols* and *ants* is consistent with regulation of these genes being one level of control.

New Proteins at the Cell Surface

Another area of unanswered questions in myoblast fusion was at the membrane and centred on the immunoglobulin superfamily members Duf and Sns. The description and analysis of two other members of this family has necessitated a reappraisal of how Duf — also called Kirre — and Sns might function. The first gene is *roughest* (*rst*), which maps very close on the chromosome to *duf* and encodes a protein with a similar extracellular domain to Duf [11]. A chromosomal deletion that removes both genes causes a severe phenotype with no myoblast fusion [11,34]. Either *rst* or *duf* can rescue this mutant phenotype and each can act as a myoblast attractant [11,34]. However, lack of either gene alone has little effect on muscle development [11].

Despite the apparent functional redundancy shown by this work there are two notable differences between *rst* and *duf*. The first is that although they are clearly structurally related, the intracellular domains are very different in length and share only 15% amino acid identity [11]. This might affect proteins that interact with the cytoplasmic domains of Rst and Duf. The second is the pattern of expression. Notably, and in contrast to *duf*, *rst* is expressed in both founders and FCMs. Furthermore, *duf* expression is generally much more restricted than *rst* and is switched on later in development. Nevertheless, Strunkelnberg *et al.* [11] reasonably conclude, given the overlapping mesodermal expression patterns of *rst* and *duf* and the significant structural similarity between the two proteins, that *rst* and *duf* have at least partially redundant functions during muscle development.

The second new family member is *hibris* (*hbs*), which has an extracellular domain closely related to Sns and human Nephrin [12,13]. Although *hbs* is expressed more widespreadly than *sns*, it too is expressed in FCMs but not in founders, and so is another example of molecular asymmetry in fusion (Figure 1C-D). Hbs is localised at the cell membrane and has similar temporal and spatial expression to Sns, although there are differences. However, in contrast to *sns*, *hbs* mutations do not have a dramatic effect on muscle, although the precise phenotype is debated [12,13]. Nevertheless, over-expression of Hbs in the somatic mesoderm disrupts myoblast fusion. Moreover, phenotypic analysis of *hbs* and genetic interactions with *sns* suggest Hbs is a dose-dependent regulator of fusion and may antagonise Sns function [12].

Four immunoglobulin superfamily members have therefore been identified to date. Although *hbs* and *rst* are expressed more widespreadly than their structural counterparts *sns* and *duf*, all four molecules must be considered in constructing a model for myoblast fusion. How do they function? Because other members of the immunoglobulin superfamily have roles in adhesion and signalling these are properties to consider closely for these four cell surface *Drosophila* proteins. The first question to ask is whether these proteins physically interact, homotypically or heterotypically. The first indication of such an interaction has recently been reported by Dworak *et al.* [13]. They find that cells transfected with *duf* aggregate with cells transfected with *sns*. This is consistent with a physical interaction between Duf and Sns that could link founder with FCM by adhesion. They present similar evidence for Duf and Hbs, but detect no interactions with *rst*-transfected cells. More detailed studies will no doubt follow both to investigate these interactions further and explore possible ligand–receptor relationships.

As described above Duf and Rst can act in the very first step of fusion as myoblast attractants (Figure 2), and this is a function that could be mediated through adhesion [4,34]. However, because no fusion at all is seen in embryos mutant for *duf* plus *rst* [34], where some FCMs will be in the vicinity of founders, it seems that these two surface proteins may also function later in the fusion process, possibly through signalling. This idea is supported by experiments demonstrating links between Duf and the cytoplasmic proteins Ants and Rols, which then link with reorganisation of the cytoskeleton. During fusion in normal development Ants redistributes from the cytoplasm to localised sites at the membrane. This does not occur in *duf*, *rst* double mutants, and the binding of Duf by Ants described earlier suggests that direct protein–protein interactions lie behind this [5].

Such interactions may also provide a link to cytoskeletal changes through Mbc, because Ants binds Mbc [5] and *mbc* mediates cytoskeletal changes [36]. Rols may also function in the same complexes or in others operating in parallel. The normal redistribution of Rols to the membrane does not occur in *duf*, *rst* double mutants, and co-overexpression of Duf with Rols results in Rols becoming enriched at the membrane [6]. D-Titin, a scaffold-like protein, is also recruited to fusion sites through membrane-associated Rols [6]. Together these findings suggest that there is a cascade of events operating from the surface proteins in founders that leads to the formation of specialised sites along the membrane required for fusion that in turn link to cytoskeletal organisation.

Duf and Sns Outside Somatic Muscle

When *duf* and *sns* were first described a pressing question was whether they functioned in cell fusions apart from in *Drosophila* somatic muscle. Recent work shows that they do [14,15]. The system investigated was *Drosophila* midgut visceral muscle, which is a latticework of longitudinal and circular fibres that encloses the midgut endodermal tube. The initial studies on *duf* and *sns* reported expression of these two genes in the visceral mesoderm [34,35]. This expression of two fusion genes was perplexing in that previous work and beliefs had it that *Drosophila* visceral muscle was not syncytial. However, it has now been found, using both dye-filling of cells and the elegant technique of Klapper *et al*. described above, that both the longitudinal and circular fibres are actually small syncytia [10,14,15]. These groups then addressed the role of *duf* and *sns* in the development of the midgut visceral muscle [14,15]. It, like the somatic muscle, was found to be organised with founders that express *duf* and fusion competent cells that express *sns*. Importantly, in mutations either of *duf* plus *rst*, or of *sns*, the two populations remain distinct and do not closely adhere and fuse as they would in the wild type.

Concluding Remarks

In the last year many more players in the multistep process of myoblast fusion have been identified. Progress in defining what these proteins do, and when do they do it, will require a detailed analysis of the cell biology, including at an ultrastructural level, of each mutant. Further studies will also establish the extent to which both the molecules and the developmental strategy of myoblast fusion in *Drosophila* are conserved in other species. Certainly the finding and initial analysis of mammalian *ants* and *mbc* suggests that elements will be conserved [5,36,40].

Another area of particular interest will be defining any spatial and temporal heterogeneity in the complexes containing immunoglobulin superfamily members. This will impact on two areas of myoblast fusion: the asymmetry of the process and the emerging idea that not all fusions are equal. There are certainly spatial differences (Figure 1C-D). There may be surface complexes containing Duf and Rst on founders and Sns, Hbs and Rst on the FCMs, and untangling the relative contributions of Duf and Rst will be one challenge. However, the asymmetry of fusion is still not understood. Although Duf expression is founder-specific, fusion can still occur without asymmetric expression of Duf, or Rst [11,34]. The answer may lie in the asymmetric assembly of functionally distinct complexes in which other asymmetrically distributed proteins have a role.

Establishing whether there is also temporal heterogeneity in the cell surface complexes is important. This could be manifested in the founders or the FCMs, which certainly appear to be heterogeneous in aspects of their differentiation, and might indicate that some myoblast fusions are indeed different from others. All this lies in the future. For now it is apparent that the current phase of gene identification and initial analysis will continue for some time yet and then the challenge will be to integrate this knowledge to produce an all embracing molecular and cellular picture of myoblast fusion.

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